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The genetics of chemoreception in the labella and tarsi of *Aedes aegypti*

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ABSTRACT

The yellow-fever mosquito Aedes aegypti is a major vector of human diseases, such as dengue, yellow fever, chikungunya and West Nile viruses. Chemoreceptor organs on the labella and tarsi are involved in human host evaluation and thus serve as potential foci for the disruption of blood feeding behavior. In addition to host detection, these contact chemoreceptors mediate feeding, oviposition and conspecific recognition; however, the molecular landscape of chemoreception in these tissues remains mostly uncharacterized. Here we report the expression profile of all putative chemoreception genes in the labella and tarsi of both sexes of adult *Ae. aegypti* and discuss their possible roles in the physiology and behavior of this important disease vector.

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1. Introduction

Aedes aegypti (L.) (Diptera: Culicidae) mosquitoes contribute to the spread of dengue, yellow fever, chikungunya and West Nile viruses through blood meal-mediated viral transmission. The global health risks associated with this disease vector are increasing (Bhatt et al., 2013; Guzman et al., 2010; Barrett and Higgs, 2007), as these mosquitoes thrive in urban environments. With potential vaccines still in development (Wallace et al., 2013), bite prevention is essential to curb the spread of these deadly diseases.

Disruption of mosquito host-seeking and feeding behavior has been achieved through use of several repellent chemicals like DEET and Picaridin (Dickens and Bohbot, 2013); and while progress has been made towards understanding the molecular associations of these repellents, their mechanisms of action remains unclear. The repellency response appears to involve multiple chemoreception pathways. *Ae. aegypti* avoidance of volatile DEET is mediated by the Odorant Receptor gene family, a molecular pathway also involved in DEET perception in the dipteran relative *Drosophila melanogaster* (Ditzen et al., 2008; DeGennaro et al., 2013). Transgenic *Ae. aegypti* lacking this olfactory pathway will land on DEET treated human skin, but will not blood-feed after contact with DEET, suggesting that non-olfactory molecular pathways of the tarsi, labella or elsewhere are involved in DEET sensation and behavioral avoidance (DeGennaro et al., 2013). Recently, Ionotropic Receptors have also been shown to play a role in DEET avoidance in *D. melanogaster* (Kain et al., 2013), highlighting the need to investigate the role of all known insect chemoreception gene families in the avoidance of repellents by mosquitoes.

Interestingly, DEET avoidance by *D. melanogaster* in feeding assays requires at least three Gustatory Receptors (Lee et al., 2010). In *Ae. aegypti*, bitter sensing gustatory receptor neurons (GRNs) of the labella respond to DEET and other repellents in electrophysiological recordings (Sanford et al., 2013), but this response's effect on mosquito behavior is unknown. Thus, putative chemoreception genes expressing in the GRNs of the major gustatory appendages may serve as useful targets to develop novel deterrents.

Several classes of genes involved in chemoreception have been identified in insects: gustatory receptors (*Grs*) (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003), odorant receptors (*Ors*) (Clyne et al., 1999; Vosshall et al., 1999; Robertson et al., 2003), ionotropic receptors (*Irs*) (Benton et al., 2009; Croset et al., 2010), odorant binding proteins (*Obps*) (Vogt and Riddiford, 1981; McKenna et al., 1994; Galindo and Smith, 2001), sensory neuron membrane proteins (*Snmps*) (Rogers et al., 1997; Vogt et al., 1999; Benton et al., 2007; Jin et al., 2008),

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14. ABSTRACT

The yellow-fever mosquito Aedes aegypti is a major vector of human diseases, such as dengue, yellow fever chikungunya and West Nile viruses. Chemoreceptor organs on the labella and tarsi are involved in human host evaluation and thus serve as potential foci for the disruption of blood feeding behavior. In addition to host detection, these contact chemoreceptors mediate feeding, oviposition and conspecific recognition; however, the molecular landscape of chemoreception in these tissues remains mostly uncharacterized. Here we report the expression profile of all putative chemoreception genes in the labella and tarsi of both sexes of adult Ae. aegypti and discuss their possible roles in the physiology and behavior of this important disease vector.

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pickpocket channels (*ppks*) or DEG/ENaCs (Liu et al., 2003), transient receptor potential channels (*Trps*) (Al-Anzi et al., 2006; Kwon et al., 2010; Kim et al., 2010; Kang et al., 2010) and CheA/Bs (Xu et al., 2002; Park et al., 2006). These genes are typically expressed in chemosensory neurons or support cells associated with peripheral hair-like sensory organs called sensilla. These chemosensory neurons discriminate between diverse chemicals, often informing mosquito behaviors such as host-seeking, feeding, oviposition and mating.

There are few comprehensive reports of chemoreception gene expression in specific appendages in dipteran species; confirmation of expression in *D. melanogaster* has been shown through non-quantitative visual representations using promoter-driven reporters or amplified PCR products, with these reports being organized by chemoreception gene family rather than global expression profile (Vosshall et al., 1999; Galindo and Smith, 2001; Couto et al., 2005; Benton et al., 2009; Isono and Morita, 2010). In *Ae. aegypti*, characterization of *Or* expression has been conducted non-quantitatively by PCR in all head appendages (Bohbot et al., 2007), and three *Grs* have been studied in the maxillary palps (Erdelyan et al., 2012; Bohbot et al., 2013). We previously reported the expression profile of *Grs* in the labella and tarsi of male and female *Ae. aegypti* (Sparks et al., 2013). Here we extend our survey to the expression of other putative chemoreception genes by RNA-seq in these tissues, thus expanding our knowledge of the appendage-specific molecular components potentially involved in chemical attraction and avoidance. Expressed putative chemoreception genes in male and female mosquitoes may be targeted in future experiments aimed at altering their behavior and ultimately disrupting transmission of harmful viruses. Furthermore, we assessed the significance of this expression for a small set of chemoreception genes by comparing RT-qPCR estimates of expression between female labella and female carcass tissue samples.

2. Materials and methods

2.1. Animal rearing

Ae. aegypti eggs (Orlando strain) were obtained from the Center for Medical and Veterinary Entomology, USDA, ARS in Gainesville, FL, USA. Larvae were reared at 25 °C (12-hL:12-hD) and fed with ground TetraMin® fish food. Unsexed pupae were hand-collected daily and transferred to plastic dishes (9 cm × 5.5 cm) inside small containment buckets, thus establishing 24-h age groups. Greater than 95% of adults emerged 2 days post-pupation, after which all remaining pupae were removed from containment buckets. Adult mosquitoes were fed with a 10% sucrose solution and maintained in an environmental chamber at 27 °C and 70% relative humidity under the same photoperiod as larvae. Tissues used in our studies were collected during the photophase from adult mosquitoes 6–7 days old.

2.2. RNA isolation and sequencing

For RNA sequencing, paired labella from 500 males or 500 females were carefully dissected to limit inclusion of other adjacent proboscis tissues. Samples from legs were comprised of pro-, meso-, or metathoracic tarsal segments of 400 males or 400 females. Dissected tissues were immediately stored on dry ice and mechanically ground in TRIzol® (Life Technologies, Carlsbad, CA, USA). Total RNA was isolated by RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA), quantified on a Nanodrop ND-1000 spectrophotometer (Nano Drop Products, Wilmington, DE, USA), and sent to the Genomics Services Lab at the Hudson Alpha Institute for Biotechnology (Huntsville, AL). Messenger RNA isolation and subsequent cDNA synthesis were completed using NEBNext® reagents (NEB, Ipswich, MA, USA) and standard protocols with custom GSL adaptors. Complementary DNA libraries corresponding to distinct tissues were sequenced on an Illumina HiSeq2000 to generate 25 million 50 base pair, paired-end reads per sample.

2.3. Analysis of annotated and unannotated chemosensory genes

Reference genome and annotations for *Ae. aegypti* (AaegL1.3) were downloaded from VectorBase (http://aaegypti.vectorbase.org/GetData/Downloads/). Output Fastq Illumina files were mapped to the reference genome with TopHat (Trapnell et al., 2009). The unambiguous sequence alignment files were uploaded into the Avadis NGS software (Strand Scientific Intelligence, CA, USA), where quantification and normalization were performed. Prior to quantification using the 'Deseq' normalization method, the read list was filtered to remove duplicate, single-end, mate-filtered, mate-missing, one-mate flip, both-mate flip, and unaligned reads. Read quality metric values were as follows: Quality threshold ≥30, N's allowed in read ≤0, Alignment score threshold ≥95, Mapping quality threshold ≥40. Transcript expression levels for all genes are reported in values of Reads Per Kilobase per Million reads mapped (RPKM). RPKM values represent a quantitative measure of the number of corresponding 50 bp sequence reads (sequenced in both directions) for a given gene. We assigned no specific RPKM threshold for functional expression vs. background "noise."

2.4. Quantitative RT-PCR

Thirteen chemosensory genes and one housekeeping gene were selected for qPCR analysis to evaluate gene expression over a dynamic range, both in copy number and presumed chemosensory gene function. Primer pairs were designed for each target gene to amplify a specific 100–180 base pair PCR product (Primer-BLAST Primer Designing tool, NCBI). At least one primer per set spans an exon boundary to exclude non-specific gDNA amplification.

Statistically supported RT-qPCR validation of RNA sequencing of labella and all tarsal types of both sexes was previously reported in Sparks et al. (2013). Here we dissected 150 paired female labella and 10 female carcasses (thorax, halteres and abdomen) for RNA extraction, repeating each tissue once. Total RNA was isolated from all frozen tissue samples as previously described. cDNA was synthesized using Superscript® III First-Strand Synthesis Supermix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). PCR products were directly sequenced to confirm amplicon identity (data not shown) (Macrogen, Rockville, MD, USA). RT-qPCR was subsequently performed on each target gene using KiCqStart® SYBR® Green qPCR ReadyMix™ iQ (Sigma–Aldrich, St. Louis, MO, USA) and an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). All Ct values were calculated by Bio-Rad iQ5 Optical System Software (Bio-Rad, Hercules, California, USA). Reactions were performed in technical triplicate 20 μL volumes. Three-step cycles plus melt curves were used for each reaction, using an annealing temperature of 56 °C for all primers. Efficiencies for each primer set were calculated from the slope of the standard curve using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001; Rasmussen, 2001). Primer efficiencies are based on three 1:10 serial dilutions of cDNA template used in side-by-side technical triplicate reactions. Efficiencies are listed in Table S1.

Relative gene quantification was calculated as $E_{\text{target}}^{-(Ct[\text{target}]-Ct[\text{reference}])}$ for each target gene (14 total) and averaged for each replicate (6 total for each tissue sample, representing two biological replicates). *Ae. aegypti* housekeeping gene Lysosomal Aspartic Protease (Vectorbase ID: AAEL006169) was used to normalize Ct values between tissue types. 'Times-enrichment' was calculated as

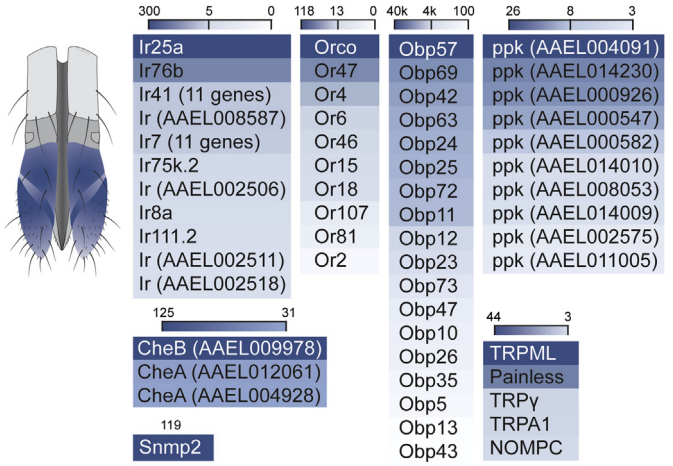

Fig. 1. Prominent chemoreception genes of the labella. Each colored cell represents the average RPKM value between male and female labellar samples (tissue collected in blue). Only annotated chemoreception genes yielding averages above 3 RPKM are listed except for *Obps*, which are at least 100 RPKM. Individual heat map scales are shown to highlight the most abundantly expressing genes within each gene family. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the ratio of female labella transcript abundance over female carcass transcript abundance.

3. Results

Here we report the labellar and tarsal expression of genes associated with chemoreception that potentially affects behaviors such as host-seeking, feeding, oviposition and mating. Gene expression is reported as RPKM, which denotes relative transcript abundance for each putative chemoreception gene. For a small selection of chemoreception genes, we also quantify relative abundance in the labella with respect to carcass. We highlight possible functions for these genes drawing on functionally characterized insect homologs.

3.1. Odorant Receptors (Ors)

Insect ORs function as heterodimers to form ligand gated ion channels (Sato et al., 2008; Wicher et al., 2008), with single ORs conferring odor sensitivity to individual odorant receptor neurons (ORNs). Our survey showed expression of the ubiquitous co-receptor *AaegOrco* and several putative ligand-binding *AaegOrs* in male and female labella (Fig. 1 and S1); RPKM values for *AaegOrco* were similar for both sexes (123.8, male labella; 112.2, female labella). Six other *AaegOrs* showed RPKM values above 10.0 (*AaegOr4*, *AaegOr6*, *AaegOr15*, *AaegOr18*, *AaegOr46* and *AaegOr47*) and three *Ors* showed intermediate RPKM values between 3.3 and 8.4 (*AaegOr2*, *AaegOr81* and *AaegOr107*) supporting previous reports of *Or* expression in the proboscis of mosquitoes (Pitts et al., 2004; Melo et al., 2004; Kwon et al., 2006; Bohbot et al., 2007). Interestingly, there was low-level (RPKM between 2.2 and 5.5) expression of a few putative ligand-binding *Ors* in the tarsi (*AaegOr2*, *AaegOr52*, *AaegOr62* and *AaegOr107*), but no *Orco* expression above 0.5 RPKM (Fig. 2 and S1). As insect ORs likely ubiquitously require ORCO co-expression for functional activation and dendritic trafficking (Larsson et al., 2004; Jones et al., 2005; Benton et al., 2006), it is unlikely *AaegOr* expression in the tarsi significantly contributes to chemoreception in this context.

Two *AaegOrs* that express in labella of *Ae. aegypti* are highly similar in sequence to an *Or* from the malaria mosquito *Anopheles*

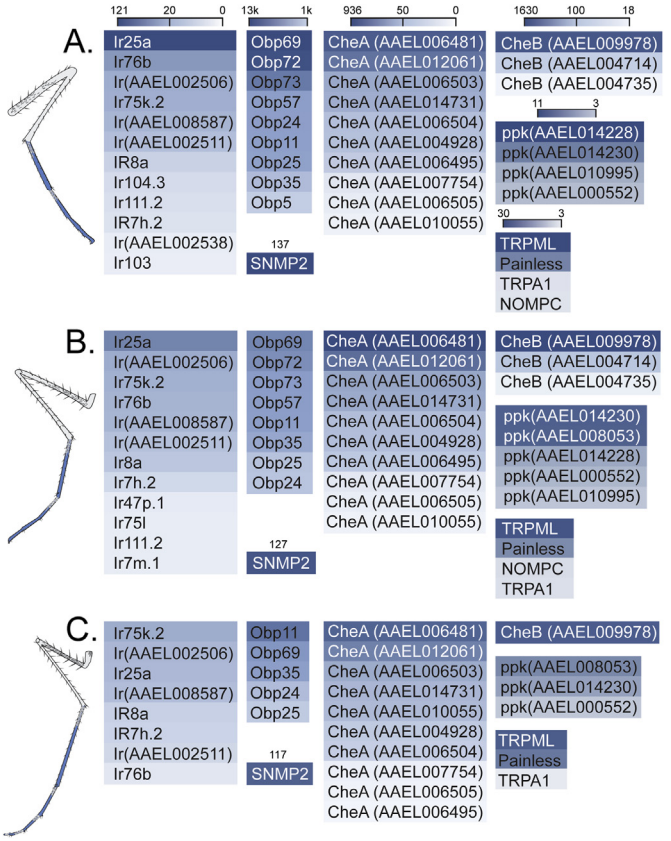

Fig. 2. Prominent chemoreception genes of the pro- (A), meso- (B) and meta-thoracic (C) tarsi. Each colored cell represents the average RPKM value between male and female tarsal samples (tissue collected in blue). Only annotated chemoreception genes yielding averages above 3 RPKM are listed except for *Obps*, which are at least 1000 RPKM. A full list of annotated chemoreception gene expression is available in the supplementary information. Individual heat map scales are shown to highlight the most abundantly expressing genes within each gene family for the three tissue types. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gambiae. *AaegOr4* and *AaegOr6* are closely related to the *AgamOr6* (Bohbot et al., 2007), which is tuned to detect several naturally occurring aromatics like acetophenone and heterocyclic compounds (Carey et al., 2010) and is expressed in the proboscis of *Anopheles gambiae* (Kwon et al., 2006). Thus, *Aaeg*OR4 and *Aaeg*OR6 may be sensitive to similar classes of volatile chemicals in the context of the labellum. The significance of these chemicals for mosquito ecology is unclear.

While the closely related (49% identical, 69% similar) AaegOr46 and AaegOr47 receptors share no homologs with greater than 25% identity in *A. gambiae* (Bohbot et al., 2007), of the four most closely related *AgamOrs* to this *Ae. aegypti Or* subfamily (100 bootstrap support, 1000 iterations), two (*Agam*OR56 and *Agam*OR57) also show expression in *A. gambiae* whole proboscis (Kwon et al., 2006). Whether the conservation of proboscis expression between these mosquito ORs is related to function requires further investigation.

3.2. Ionotropic Receptors (Irs)

IRs are ligand-binding chemoreceptors in insects that have likely evolved from animal ionotropic glutamate receptors (Croset et al., 2010), which are a conserved family of ligand-gated ion channels associated synaptic communication in both eukaryotes and prokaryotes. Since their identification (Benton et al., 2009), IRs have been primarily described as olfactory receptors in

D. melanogaster (Abuin et al., 2011; Ai et al., 2013), although other functional roles have been assigned to IRs expressing in non-antennal tissues (Senthilan et al., 2012; Zhang et al., 2013).

Thirty-six putative *Irs* show expression levels above 3.0 RPKM in the labella and tarsi of *Ae. aegypti* (Figs. 1 and 2 and S1), which strongly suggests their involvement in chemoreception in these appendages. *AaegIr25a*, which has clear orthologs in all protostomes (Croset et al., 2010) and is the most similar IR to iGluRs, showed the highest RPKM values for labella, pro- and meso-thoracic tarsi, with RPKM values over 300 for male and female labella (Figs. 1 and 2). Both *Dmel*IR25a expression (Benton et al., 2009) and function (Abuin et al., 2011) in ORNs innervating many coeloconic sensilla on the antenna of *D. melanogaster* strongly suggest IR25a is a co-receptor for other ligand-binding IRs (Benton et al., 2009). This function is consistent with our expression data, as *AaegIr25a* is the most abundantly expressed *Ir* in our survey.

Other conserved putative *AaegIrs* (Rytz et al., 2013) showing expression in labella and/or tarsi are: *AaegIr8a*, *AaegIr76b*, the *AaegIr41* subfamily, and two members of the *AaegIr75* subfamily (Figs. 1 and 2). *AaegIr8a* shows RPKM values between 5.9 and 9.6 in male and female tarsi (Fig. 2), much lower than *AaegIr25a*. Like *Aaeg*IR25a, *Aaeg*IR8a may function as an IR co-receptor based on data from its ortholog *Dmel*IR8a (Benton et al., 2009; Abuin et al., 2011). *AaegIr76b* is abundantly expressed in the labella (RPKM reaching 153.3 in males) and prothoracic tarsi of males and females and less so in meso- and meta-thoracic tarsi. *AaegIr76b* is orthologous to *DmelIr76b*, which is involved in salt reception in *D. melanogaster* GRNs (Zhang et al., 2013), but is also expressed in all classes of coeloconic sensilla on the antenna (Benton et al., 2009). The *AaegIr41* subfamily shows RPKM values as high as 42.4 for *AaegIr41e*, and most of these thirteen *Irs* show expression above 10.0 RPKM in male and female labella. Though the *DmelIr41a/AaegIr41* group appears to be conserved throughout several insect orders (Rytz et al., 2013), this is only the second report of its expression (Leal et al., 2013). The *Ir75* subfamily in *Ae. aegypti* has expanded to ten members; two of these *Irs*, *AaegIr75k.2* and *AaegIr75l*, are expressed in labella and tarsi of males and females. *Dmel*IR75 is likely involved in propionic acid reception in the antenna of *D. melanogaster* (Yao et al., 2005; Abuin et al., 2011). Propionic acid and other carboxylic acids are mosquito attractants in the context of odor blends containing ammonia and lactic acid (Smallegange et al., 2009). Thus, *Aaeg*IR75k.2 and *Aaeg*IR75l may be involved in the detection of attractants emitted from human skin, which include several aliphatic carboxylic acids (Bosch et al., 2000).

In addition to these conserved *Irs*, eleven of the nineteen *AaegIrs* (with accession numbers) associated with the dipteran *Ir7* subfamily (Croset et al., 2010) are expressed at RPKM levels above 4.0 in male and female labella (Fig. 1). This expression is consistent with the observation that *Ir7* subfamily member *DmelIr7a* is expressed in both adult and larval gustatory neurons in *D. melanogaster* (Croset et al., 2010). Thus, it likely that at least some of the expressed *Aaeg*IRs are involved in chemical reception in the labellum.

Other putative *Irs* showing RPKM values higher than 3.0 in labella and/or tarsi are *AaegIr120*, *AaegIr111.2*, *AaegIr103*, *AaegIr104.2*, *AaegIr104.3*, and five unnamed *Irs* (Figs. 1 and 2). Among dipteran genomes, *AaegIr120* is most closely related to *CquiIr120* (Croset et al., 2010), which is more abundant in the metathoracic tarsi than the antennae of *Culex quinquefasciatus* (Leal et al., 2013). This is consistent with tarsal expression levels for *AaegIr120* observed here (Fig. 2).

Genes specific to or enriched in chemoreception organs of female *Ae. aegypti* are candidates for involvement in blood host location and recognition. There were no obvious sex biases among *AaegOrs* expressed in labella, however a few *AaegIrs* demonstrated sex biases. *AaegIr103*, *AaegIr104.2* and *AaegIr104.3* are most abundant (>4 times enriched) in female pro- and meso-thoracic tarsi and are not expressed in labella of either sex (Figure S1). The tarsi represent the initial point of contact between feeding female mosquitoes and their hosts (Clements, 1992); thus, tarsi may house female-specific chemoreceptors that first assess the non-volatile cues associated with potential blood hosts. Alternatively, these female tarsal enriched IRs may be involved in the recognition of conspecific males, a function of the tarsi of *D. melanogaster* (Greenspan and Ferveur, 2000).

3.3. Sensory neuron membrane proteins (Snmps)

Snmps are members of a gene family characterized by human CD36, having diverse functions including transport of fatty acids and interactions with other proteins in cell:cell recognition (Vogt, 2003; Silverstein and Febbraio, 2009). SNMPs are membrane proteins observed to associate with chemosensory sensilla in insects, and are conserved throughout four orders of the holometabolous lineage (Vogt et al., 2009). In *D. melanogaster*, SNMP1 is essential for the detection of the pheromone cis-vaccenyl acetate (Benton et al., 2007; Jin et al., 2008). Here we focus on the two putative *Snmps* identified in *Ae. aegypti*, *Snmp1* and *Snmp2* (Vogt et al., 2009).

*AaegSnmp*2 is abundantly expressed in the labella and tarsi of both sexes, although RPKM values are somewhat higher in males (RPKM range 140.8–168.9 for male tissue, 81.9–104.3 for female tissue; Figs. 1 and 2). This is consistent with reported *Snmp2* expression in leg and gustatory tissue from the dipteran *D. melanogaster* (Benton et al., 2007; Vogt et al., 2009) as well as the lepidopterans *Cnaphalocrocis medinalis* (Liu et al., 2013) and *Agrotis ipsilon* (Gu et al., 2013). As yet, no function has been attributed to SNMP2 in insects.

Conversely, *AaegSnmp1* showed almost no expression in labella or tarsi (maximum RPKM of 0.1 in female labella and metathoracic tarsi; Figure S1), an unexpected result considering a previous non-quantitative report of leg expression in *Ae. aegypti* (Vogt et al., 2009). It is possible that *AaegSnmp1* transcript was represented in femoral or tibial tissues in this instance, as these leg segments were not included in our survey.

Snmp1 homologs are expressed more broadly in other insect species. Relatively low expression in bodies and legs of *D. melanogaster* has been reported (Benton et al., 2007; Vogt et al., 2009). *Snmp1* expression in lepidopteran species varies from widespread, including legs, in *Plutella xylostella* (Li and Qin, 2011), *C. medinalis* (Liu et al., 2013) and *A. ipsilon* (Gu et al., 2013), to antennal-specific in other species including *A. gambiae* (Benton et al., 2007), *Manduca sexta* (Rogers et al., 2001) and *Amyelois transitella* (Leal et al., 2009).

3.4. Ion channels associated with chemoreception (Trps and Ppks)

TRP channels belong to a highly conserved family of ion channels that express and function in multiple types of sensory neurons in insects (Fowler and Montell, 2013). Here we focus on potential roles in chemoreception, although these membrane proteins are also involved in photo-, thermo-, and mechanoreception in insects. Five putative *AaegTrp* channels (Bohbot et al., 2014) show expression above 3.6 RPKM in male and female labella (*Trpa1*, *Painless*, *Trpml*, one of the two *Nompc* homolgues, and Trpγ) (Fig. 1). All but *Trpγ* also express above 4.7 RPKM in at least one tarsal sample (Fig. 2). Lower RPKM values may indicate functional significance for other putative *AaegTrp* channels (Figure S1).

*Aaeg*TRPA1, *Aaeg*Painless and *Aaeg*TRPγ are the most likely TRPs to contribute to chemoreception in labella and tarsi as homologs of these channels affect chemoreception in other insects. In

D. melanogaster, neuronal responses to citronellal in the antenna are mediated by TRPA1 (Kwon et al., 2010). While citronellal did not directly activate *D. melanogaster* TRPA1 in the aforementioned study, it did directly activate mosquito *A. gambiae* TRPA1. Thus, TRPA1 may contribute in some way to the reception of repellents by GRNs in the context of the labella and tarsi of *Ae. aegypti*. Similarly, noxious chemical electrophiles have been shown to deter feeding responses in *D. melanogaster* and evoke *Dmel*TRPA1- and *Agam*TRPA1-mediated responses when they are expressed in a heterologous system (Kang et al., 2010). Furthermore, *Dmel*TRPA1 appears to be involved in gustatory reception of aristolochic acid but not broadly for other bitter compounds (Kim et al., 2010). In the lepidopteran *Manduca sexta*, TRPA1 mediates gustatory responses to aristolochic acid and integrates information about temperature (Afroz et al., 2013), possibly a result of altered thermosensitivity through the evolution of novel isoforms to gain or maintain chemical sensitivity (Kang et al., 2012).

*Dmel*Painless is required for the gustatory-mediated avoidance of isothiocyanate (Al-Anzi et al., 2006); thus *AaegPainless* (the closest *Ae. aegypti* homolog, Bohbot et al., 2014) expression in the labella may indicate a similar sensitivity in mosquitoes.

*Dmel*TRPL, a member of the TRPC subfamily associated with phototransduction (Fowler and Montell, 2013), is also involved in the feeding adaptation to camphor in *D. melanogaster* (Zhang et al., 2013b). *Dmel*TRPL is closely related to *Aaeg*TRPγ (Bohbot et al., 2014); therefore, *AaegTrp*γ expression in the labella may indicate involvement in gustatory perception.

Ppk channels are members of the diverse animal-specific DEG/ENaC family of ion channels. While poorly understood in insects in general, *D. melanogaster* Ppks are spatially and temporally diverse in their expression pattern (Zelle et al., 2013), and are involved in the gustatory detection of salt (Liu et al., 2003) and mating cues (Lin et al., 2005; Thistle et al., 2012; Lu et al., 2012; Toda et al., 2012; Starostina et al., 2012). We observed moderate expression (RPKM range 3–27) of 10 *Aaegppks* in labella and 7 *Aaegppks* (RPKM range 3–11) in tarsi (Figure S1). Of these genes, only 2 *Aaegppks* are expressed in both tissue types. We also detected low *Aaegppk* expression (RPKM 1–3) in multiple instances (Figure S1), which may also indicate functional significance in a restricted number of cells.

3.5. Small, soluble proteins associated with chemoreception (Obps and CheA/Bs)

Insect OBPs are small soluble proteins, many of which are excreted into the lymph surrounding chemoreceptor neurons and influence the detection of both volatile (Vogt and Riddiford, 1981; Vogt et al., 1999) and non-volatile compounds (Jeong et al., 2013; Swarup et al., 2013). The *Ae. aegypti* genome contains 111 *Obp* genes subdivided into three subfamilies that have been defined based on bioinformatics data (Zhou et al., 2008; Manoharan et al., 2013). Classic OBPs have a conserved motif of 6 cysteine residues. Plus-C OBPs possess additional cysteine residues, and two-domain (also referred to as “atypical”) OBPs are the largest OBP subfamily.

AaegObps showed the highest level of transcript abundance in our survey (RPKM range 0–45,000; Fig. 3). While there are some differences in expression levels between sexes (Figure S1), sex-biased expression of *Obps* likely does not account for physiological or behavioral differences between male and female *Ae. aegypti*. The most abundantly expressed *AaegObps* in the labella of both sexes are *Obp57*, *Obp69*, *Obp42*, *Obp63*, *Obp24*, *Obp25*, *Obp72* and *Obp11* (RPKM range 3500–45,000; Figs. 1 and 3). Some *Obps*, like *Obp5*, *Obp11*, *Obp35* and *Obp81*, were more abundant in specific tarsal types than in labella, often a feature shared between sexes for specific genes. In all, 18 *Obps* showed RPKM values greater than 100 in labella and 16 showed RPKM values above 100 in at least one tarsal tissue (Figure S1). The two-domain *Obps* are generally not expressed in labella or tarsi (Fig. 3), possibly highlighting a functional difference in this sub-family of *Obps*.

Functional data on mosquito OBPs is lacking. The *AaegObp1* homolog *An. gambiae Obp1* has been reported to specifically bind to the oviposition attractant indole (Biessmann et al., 2010). *AaegObp1* homolog *C. quinquefasciatus Obp1* has been implicated in the sensitivity of female mosquitoes to several oviposition cues (Pelletier et al., 2010). We observed relatively little *AaegObp1* expression in the tissues we surveyed (Fig. 3). The knockdown of two *Ae. albopictus Obps* (*AalbObp37* and *AalbObp39*) affected antennal responses to indole (Deng et al., 2013). *AaegObp39*, the closest homolog of *AalbObp39*, showed moderate expression in all tissues we surveyed (Fig. 3).

Insect CheAs and CheBs represent two separate gene families that encode small, membrane-bound proteins that are excreted in some cases (Xu et al., 2002; Ben-Shahar et al., 2010). These genes are distinct from *Obps* though they too are usually associated with chemoreceptor organs. Some CheA and CheB proteins are expressed in sex-specific patterns and are thought to mediate pheromone reception in sex-specific tarsal sensilla of *D. melanogaster* (Xu et al., 2002; Park et al., 2006). The molecular mechanism of CheA and CheB function remains unknown, but may involve interaction with other membrane bound receptors or Ppk channels (Ben-Shahar et al., 2010).

As in *D. melanogaster*, most *AaegCheA* and *AaegCheB* genes express in tarsi in a sex-specific manner. These genes likely influence mating behavior by selectively binding to and transporting non-volatile pheromones required to initiate successful copulation (Xu et al., 2002; Park et al., 2006). Of ten putative *AaegCheA* genes (gene curation, Bohbot et al., 2014), four are highly enriched in male tarsi with respect to female tarsi and one is highly enriched in female tarsi with respect to male tarsi (Figure S1). The remaining five *Aaeg*CheA genes are abundant in tarsi of both sexes. All three putative *Aaeg*CheB genes are enriched in male tarsi with respect to female tarsi, or the labella of both sexes (Figure S1).

3.6. Enrichment of chemoreception genes in female labella

Enrichment of gene transcripts in the labellum with respect to carcass supports their involvement in chemoreception. We compared the expression of 14 genes through RT-qPCR of female labella- and female carcass-derived cDNA libraries (Fig. 4); relative abundance of each gene is presented as the ratio of labella to carcass expression. As expected, the two genes showing near zero RPKM values for female labella RNA-seq samples (*Aaeg*GR1, 0.2; *Aaeg*GR76, 0.0) also showed the lowest enrichment in female labella tissue by RT-qPCR (*Aaeg*GR1, 1.1-times; *Aaeg*GR76, 4.8-times). All other putative chemoreception genes in this target group showed relatively high enrichment, between 13 and 15,000-times, in female labella and correspond to relatively high RPKM values (Fig. 1 and S1; Sparks et al., 2013). These consistencies support the assumption that putative chemoreception gene transcripts yielding positive RPKM values above 1.0 in labella and tarsi are expressed and functional in these organs.

4. Discussion

Mosquito behaviors such as host-seeking, feeding, oviposition and mating are influenced by sensory inputs from labella and tarsi (Clements, 1992). These appendages harbor uniporous chemosensory sensilla as well as sensors of physical stimuli like heat or mechanical force (McIver and Siemicki, 1978; Lee and Craig, 2009).

Classic

	Labella	Tarsi
Obp1	1.6	2.6
Obp2	0.0	0.1
Obp3	0.0	0.1
Obp4	39.4	0.4
Obp8	17.7	32.3
Obp9	3.6	9.1
Obp10	473.5	560.2
Obp11	3963.3	4213.3
Obp12	1236.3	74.2
Obp13	122.3	72.5
Obp14	24.9	15.8
Obp15	2.8	5.4
Obp17	1.8	0.3
Obp18	0.0	0.2
Obp19	0.0	0.0
Obp20	4.6	8.4
Obp21	0.9	1.8
Obp22	1.3	0.6
Obp27	7.4	20.9
Obp34*	0.4	108.3
Obp35	338.1	1947.5
Obp36	0.3	0.0
Obp37	0.0	0.1
Obp38**	0.0	1.2
Obp39	42.3	82.6
Obp55	0.0	0.3
Obp56	8.6	1.9
Obp57	43321.6	1906.6
Obp59	3.3	1.2
Obp60	0.0	0.0
Obp61	4.8	1.4
Obp65	0.2	0.4
Obp76	15.9	54.8
Obp77	0.3	1.4
Obp78	0.0	0.1
Obp79	0.0	0.0
Obp80	0.0	0.0
Obp81	6.5	155.2
Obp83	2.0	0.5

Plus-C

	Labella	Tarsi
Obp5	243.0	500.4
Obp23	1035.2	166.9
Obp24	7278.7	1689.0
Obp25	5888.9	1483.5
Obp26	479.3	35.7
Obp42	12585.2	91.9
Obp43	104.2	1.4
Obp47	605.4	3.7
Obp48	0.2	0.1
Obp49	0.0	0.0
Obp50	0.0	0.0
Obp51	11.3	0.1
Obp52	0.0	0.0
Obp53	0.0	0.0
Obp54	0.1	0.0
Obp62	43.3	0.9
Obp63	8900.9	78.3
Obp67	0.0	0.0
Obp68	0.0	0.0
Obp69	17986.3	7262.1
Obp70	6.0	0.6
Obp71	0.9	0.0
Obp72	3861.1	5700.1
Obp73	785.1	3289.7
Obp74	0.5	0.0
Obp75	0.0	0.1
Obp82	0.0	0.2

Two-Domain

	Labella	Tarsi
Obp6	0.0	0.3
Obp7	0.6	0.1
Obp16	0.0	0.0
Obp28	0.2	0.0
Obp29	0.0	0.0
Obp31	0.0	0.0
Obp32	0.0	0.0
Obp33	0.0	0.0
Obp40	0.0	0.0
Obp41	0.0	0.0
Obp44	0.0	0.0
Obp45	0.0	0.0
Obp46	0.0	0.0
Obp58	0.0	0.1
Obp84	0.0	0.1
Obp85	0.0	0.0
Obp86	0.0	1.0
Obp87	0.0	0.0
Obp88	0.0	0.1
Obp89	0.0	0.0
Obp90	0.0	0.0
Obp91	0.0	0.0
Obp92	0.0	0.0
Obp93	1.1	0.5
Obp94	0.0	0.0
Obp95	0.0	0.0
Obp96	0.0	0.0
Obp97	0.0	0.0
Obp98	0.0	0.0
Obp99	1.7	3.4
Obp100	0.0	0.1
Obp101	0.0	0.0
Obp102	0.0	0.0
Obp103	0.0	0.4
Obp104	0.2	0.2
Obp105	0.3	0.5
Obp106	0.0	0.0
Obp107	0.0	0.1
Obp108	0.0	0.0
Obp109	0.0	0.0
Obp110	0.0	0.0
Obp111	0.4	0.1
Obp112	0.3	0.0
Obp113	1.4	3.5
Obp114	0.0	0.0

Fig. 3. Obp expression in labella and tarsi. Numbered values represent the average RPKM values for male and female labella or male and female tarsi. Averages for tarsi include all three tarsal types: pro-, meso- and meta-thoracic. Heat map coloration is capped at 30,000 RPKM with 10% intensity at 50 RPKM. *AaegObps* are divided into three columns representing classic OBPs, plus-C OBPs, and two-domain OBPs. (*) indicates homology to *D. melanogaster* LUSH; (**) indicates homology to *D. melanogaster* OSE/OSF (Zhou et al., 2008; Manoharan et al., 2013).

4.1. Olfactory genes in gustatory organs

It remains unclear with which sensory neurons the *Aaeg*ORs and *Aaeg*IRs associate in the labellum and tarsi of mosquitoes. A previous report showed ORCO immunoreactivity of a few chemoreceptor sensilla on the labella of *Ae. aegypti* (Melo et al., 2004) suggesting that *Aaeg*ORs function in the context of gustation. Additionally, multiple non-ORCO *Or* transcripts have been amplified from single gustatory sensilla on the labella from *An. gambiae* (Kwon et al., 2006), from which at least a few sensory neurons projected to the antennal lobe (Kwon et al., 2006). Sensilla on the labellum of *Ae. aegypti* and other mosquitoes have been described as either uniporous or aporous (McIver and Siemicki, 1978; Lee and Craig, 2009), which suggests that these volatile-sensing ORs and IRs may function in neurons housed in sensilla that are not multiporous, as occurs on the antennae and maxillary palps. The sensitivity of specific *Aaeg*ORs and *Aaeg*IRs in gustatory hairs of the labella and tarsi to volatile odorants requires future investigation in *Ae. aegypti*.

The elongated proboscis of mosquitoes allows them to feed on floral sugar sources as well as vertebrate hosts (Clements, 1992). Has expression of olfactory receptors like ORs and IRs in the labella co-evolved with the elongation of the mosquito proboscis? It has been shown in *An. maculipennis* that labella are dispensable for normal blood-feeding behavior (Robinson, 1939), but the opposite conclusion was made for *Ae. aegypti* in a later study (Jones and Pilitt, 1973). Targeted knockdown of olfactory receptor genes specifically in proboscis tissue may help elucidate the effect on behavior of ORs and IRs in the labella of *Ae. aegypti*.

DEET has been shown to block *Ae. aegypti* host-seeking behavior through an ORCO-mediated pathway (DeGennaro et al., 2013), and we have shown labellar expression of both *Orco* and odorant activated *Ors*; therefore, it is possible there exists a labellar component to this ORCO-mediated avoidance of volatile DEET. Whether or not ORs are involved in the physiological response to DEET by GRNs of the labella (Sanford et al., 2013) requires direct investigation. In addition to serving as an ion channel (Sato et al., 2008; Wicher et al., 2008), ORCO trafficking of conventional ORs is necessary for proper dendritic localization of ORs (Larsson et al., 2004). Perhaps ORCO may participate in the trafficking of other membrane proteins or function downstream of non-OR receptor events in the labella.

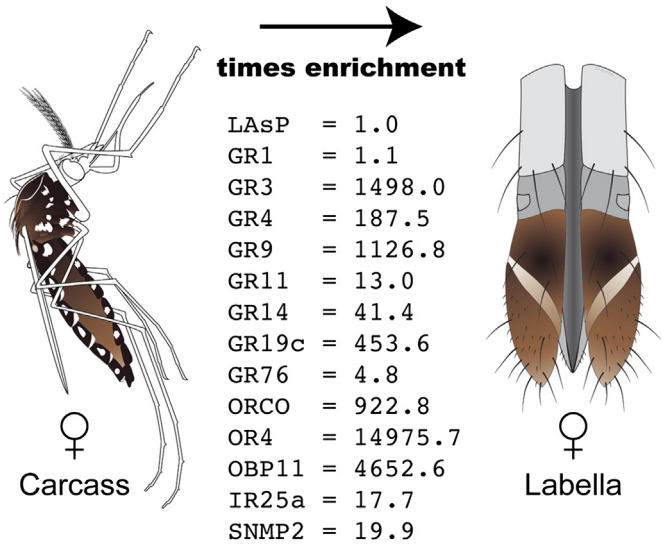

Fig. 4. Chemoreception gene enrichment in labella and tarsi. RNA samples used for RT-qPCR were obtained from the tissues depicted in color. Each gene is reported in 'times enrichment' as a ratio of relative abundance of female labella to female carcass. Each gene was first normalized to housekeeping gene *Aaeg*LAsP within the given tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Of those species surveyed by intensive sequence search analysis, *Ae. aegypti* presents the highest number of putative *Irs* (95 genes). Only a fourth of the *DmelIrs* identified by sequence homology are expressed in the antennae (Rytz et al., 2013), and evolutionary analysis of IRs throughout protostomes suggests that the function of species-specific IRs (those representing species-specific gene expansions) extend beyond olfaction to other sensory modalities (Croset et al., 2010). While IRs confer odor sensitivity to specific *D. melanogaster* ORNs and are the defining molecular feature of a large subset of antennal ORNs (Benton et al., 2009), IRs may also be sensitive to non-volatile stimuli. Expression of this ancient family of receptors in chemoreceptive tissue extends to aquatic protostomes (Croset et al., 2010); therefore, IR involvement in the reception of non-volatile compounds very likely represents an ancestral function of these receptors. Deciphering whether the labellar or tarsal *AaegIrs* identified in our survey respond to volatile and/or non-volatile compounds is worthy of future consideration. The question remains too as to whether all insect IRs are chemoreceptors. Thirty *AaegIrs* show expression levels above 3.0 RPKM in male or female labellar tissue samples, slightly more than the twenty-four *AaegGrs* expressing above this threshold in our previous report (Sparks et al., 2013). Sixteen *AaegIrs* show expression levels above 3.0 RPKM in at least one male or female tarsal tissue sample, as opposed to only eight *AaegGrs* expressing above this threshold (Sparks et al., 2013). Thus, *Irs* may mediate many of the gustatory signals affecting behavior of *Ae. aegypti*.

Although an IR-mediated DEET avoidance pathway has recently been identified in the antennae of *D. melanogaster* (Kain et al., 2013), we did not observe the expression of the homolog *AaegIr40a* in labella or tarsi (Figure S1). However, as at least thirty-six putative *Irs* are expressed in the labella and/or tarsi of female *Ae. aegypti* (Figure S1), they may be involved in detection of repellents like DEET and could provide potential targets for behavioral disruption.

The recent finding that OBPs affect responses of insect GRNs (Jeong et al., 2013; Swarup et al., 2013) is not surprising given their promiscuous expression in non-olfactory tissues (Figs. 1 and 2) as demonstrated in our survey. Based on their lack of expression, Two-Domain OBPs do not appear to affect gustatory inputs in the labella or tarsi adult *Ae. aegypti* (Fig. 3). The Two-Domain OBP *AaegObp45* is expressed and accumulates in ovaries, and is thought to mediate eggshell formation (Costa-da-Silva et al., 2013). As demonstrations of insect OBP function including specific ligands or other functional data is limited (Grosse-Wilde et al., 2006; Syed et al., 2006; Forstner et al., 2009), *Aaeg*OBP homology does not inform possible functionality. A few *Aaeg*OBPs are homologous to well-studied *Dmel*OBPs (LUSH and *Aaeg*OBP34, OSE/OSF and *Aaeg*OBP38, Manoharan et al., 2013; Fig. 3), but speculation as to their function in mosquito species requires further investigation.

4.2. Conclusions

We have revealed the expression of *Ors*, *Irs*, *Snmp2*, *Trp* channels, *Ppk* channels, *Obps*, *CheAs* and *CheBs* in the main gustatory appendages of *Ae. aegypti*, genes that serve as useful targets towards the behavioral disruption of this important disease vector. Many genes not discussed here show low, but detectable levels of expression (<3.0 RPKM, Figure S1). These genes may be functional at low copy level or may be active in a relatively small subset of cells present in the tissue we surveyed. Some chemoreception genes may be more actively transcribed at different animal ages or life stages. Of the genes assessed by RT-qPCR, all with RPKM values greater than 1 are highly-enriched in labellar tissue with respect to carcass tissue, suggesting that most of, if not all, the genes identified in our survey are involved in chemoreception. The next steps in characterizing the chemoreception capability of the labella and tarsi of *Ae. aegypti* include determining the sensitivities of specific receptors (GRs, ORs, and IRs), assessing the relationship of these receptors to putative ion channels like Trps and Ppks, and elucidating the function of highly expressed accessory genes like *Snmp2* and the most abundant *Obps* and *CheA/Bs*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2014.02.004.

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